SUPPLEMENT ARTICLE

Diversity of Rotavirus VP7 and VP4 Genotypes in Northwestern Nigeria

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Background. Nigeria has recently been ranked third among the 10 countries with the greatest number of rotavirus disease–associated deaths per year. Estimates attribute up to 33,000 deaths annually to rotavirus disease in Nigerian children <5 years old. Although the introduction of the new oral, live attenuated rotavirus vaccines may not occur for another 4–6 years in developing countries, background data on burden of disease, cost of rotavirus disease, and characterization of circulating strains is required to hasten this introduction to children who would clearly benefit from the intervention.

Methods. Between July 2002 and July 2004, fecal specimens were collected from 869 infants and young children <5 years of age presenting with diarrhea in Kaduna, Kebbi, Sokoto, and Zamfara states in northwestern Nigeria. In addition, 194 control specimens were also collected from children matched for age. Specimens were screened for the presence of rotavirus antigens. Rotavirus-positive specimens were further analyzed to determine electropherotype, subgroup specificity, and G and P genotypes.

Results. Rotavirus was detected in 18% of children with diarrhea and 7.2% of the age-matched case control subjects. The highest rotavirus burden was detected in children aged <6 months. The majority of the rotavirus-positive specimens revealed viruses of long electropherotypes, subgroup II specificity, and G1P[8] genotypes. Furthermore, more than a quarter of specimens (37%) displayed mixed G and P genotypes, and almost a third could not be genotyped.

Conclusions. The high numbers of mixed rotavirus infections highlight the multitude of enteric pathogens to which children in African countries are exposed. Data on circulating rotavirus strains serve to inform African government officials to the serious health threat posed by rotavirus in their respective countries and to document the diversity of strains before vaccine introduction.

Diarrheal diseases remain one of the foremost health burdens in many developing countries. Rotavirus has long been acknowledged to be a major etiological agent of acute gastroenteritis and responsible for a large proportion of morbidity and mortality associated with diarrheal illnesses [1]. Recent estimates generated by Parashar and colleagues [2] attribute 527,000 deaths to rotavirus annually in children <5 years, with 145,000 occurring in sub-Saharan Africa [3]. Improvements in sanitation and the availability of clean water have not decreased the rate of rotavirus diarrhea in developed countries, and the development and implementation of an effective vaccine into the routine Expanded Program on Immunization schedule is considered the first strategy of prevention [4]. The availability of rotavirus vaccines to children in developing countries may provide a new tool to address childhood mortality in African settings [5].

The rotavirus particle is made up of 3 protein layers enclosing a double-stranded RNA (dsRNA) genome. The dsRNA genome consists of 11 distinct segments of dsRNA that can be separated on a polyacrylamide gel. The resultant migration patterns or electropherotypes

Potential conflicts of interest: none reported.

Financial support: UNESCO-L'ORÉAL Fellowship for Women in Life Sciences, World Health Organization, and Medical Research Council of South Africa.

Supplement sponsorship: This article is part of a supplement entitled "Rotavirus Infection In Africa: Epidemiology, Burden of Disease, and Strain Diversity," which was prepared as a project of the Rotavirus Vaccine Program, a partnership among PATH, the World Health Organization, and the US Centers for Disease Control and Prevention, and was funded in full or in part by the GAVI Alliance.

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The Journal of Infectious Diseases 2010; 202(S1):S198–S204

0022-1899/2010/20205S1-0028$15.00
DOI: 10.1086/653570

provide a useful tool for the differentiation of strains. Electropherotypes can be broadly divided into "long," "short," "supershort," and "abnormal" RNA profiles, based on the relative migration of RNA segments 10 and 11 [6]. The 3 protein layers can be split into the core, inner capsid, and outer capsid. Group and subgroup specificity are defined by epitopes on the inner capsid protein (VP6). There are currently 7 rotavirus groups (A–G) and at least 4 subgroups (SGI, SGII, SGI+II, and SGnon-I/non-II) within group A that have been recognized.

The VP7 and VP4 proteins form the smooth outer capsid (G serotype) and short spike (P genotype), respectively, and are the major antigens inducing neutralizing immune responses during rotavirus infections. Rotavirus strains are, therefore, classified according to G and P genotypes, based on differences in epitopes on outer capsid proteins. Although 11 G serotypes and 11 P genotypes [7–15] have been detected in humans, serotypes G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] are thought to be an important cause of diarrhea in infants and young children worldwide [16].

There have been a number of published articles on rotavirus-associated disease in southern Nigeria [17–21], with a few studies conducted in northern Nigeria, predominantly in the north-eastern [22, 23] and north-central regions [24, 25]. This study expands the rotavirus data available for Nigeria and examines rotavirus strains detected between July 2002 and July 2004 in children <5 years residing in 4 states of northwestern Nigeria (Kaduna, Kebbi, Sokoto, and Zamfara). The region under study has a typical tropical continental climate and northern dry savannah vegetation with alternating seasons: humid to wet and cool (April to September) and to hot and dry (October to March). Two of the studied states (Sokoto and Kebbi) border the semiarid Niger Republic. Northwestern Nigeria has a predominantly Muslim population, comprising different educational and socioeconomic backgrounds and living in neighborhoods with distinctly different levels of sanitation. Borehole and well water are the major source of drinking water in these areas. The main occupations are cattle rearing and subsistence farming, and nomadic pastoral farmers tend to live in close association with their animals and share common sources of drinking water.

Nigeria has recently been ranked second among 6 countries with the most rotavirus disease-associated deaths per year in children <5 years old [26]. Although the introduction of the 2 new currently available oral, live attenuated rotavirus vaccines may not occur before 4–6 years in these African settings, data on burden of rotavirus disease, its cost, and characterization of circulating strains will be required to hasten vaccine implementation.

MATERIALS AND METHODS

Stool collection. Between July 2002 and July 2004, 869 stool specimens were collected from infants and young children <5 years old who presented with diarrhea at clinics or were admitted to the hospital for gastroenteritis in Kaduna, Kebbi, Sokoto, or Zamfara state in Northwestern Nigeria. During the same time period, samples were also collected from 194 age-matched case control subjects, children with diseases unrelated to gastroenteritis in the same clinics or hospitals and areas.

Stool specimens were stored frozen at −20°C and transported for further analysis to the Medical Research Council Diarrhoeal Pathogens Research Unit, University of Limpopo, Medunsa Campus, Pretoria, South Africa. On delivery, a 10% fecal suspension was prepared using distilled water, and the suspension was stored at 4°C.

Rotavirus detection. Rotavirus antigens were detected using the 10% fecal suspensions previously prepared and a commercially available Rotavirus IDEIA Kit (DakoCytomation), according to the manufacturer's instructions.

Polyacrylamide gel electrophoresis. All rotavirus-positive specimens were analyzed by polyacrylamide gel electrophoresis (PAGE), as described elsewhere [27]. Briefly, RNA was extracted from the 10% fecal suspensions previously prepared using phenol-chloroform deproteinization and ethanol precipitation. The extracted RNA was applied to a 3% stacking/10% resolving gel and eletrophoresed overnight at 100 V at room temperature, using a discontinuous buffer system. The dsRNA bands were visualized by silver staining, according to the method described by Herring et al [28]

Subgroup specificity (VP6). All rotavirus-positive specimens were analyzed using an "in-house" VP6 enzyme-linked immunosorbent assay, as described by Steele and Alexander [29]. Group-specific [30] and subgroup-specific monoclonal antibodies were used [31] (a kind donation from H. B. Greenberg, Stanford University).

VP7 and VP4 genotyping. The RNA of all rotavirus-positive specimens was extracted using TRIzol (Life Technologies) according to the manufacturers' instructions. The extracted RNA was resuspended in 15 μL of RNase-free water and stored at −20°C until required. The VP7 and VP4 reverse-transcription polymerase chain reaction (RT-PCR) amplification and genotyping were carried out as described elsewhere by Gouvea et al [32] and Gentsch et al [33], respectively. A P[14] primer (p4943) described by Mphahlele et al [34] was included with P-specific primers during genotyping.

RESULTS

Rotavirus detection. Rotavirus antigens were detected in 18.0% of diarrheal stool specimens (156 of 869) and in 7.2% of age-matched case control samples (14 of 194) collected in northwestern Nigeria. Analysis of the age distribution of children affected by rotavirus diarrhea indicates that the highest burden is in infants <6 months old (Figure 1). Further analysis of data from the 0–6-month-old group showed the highest

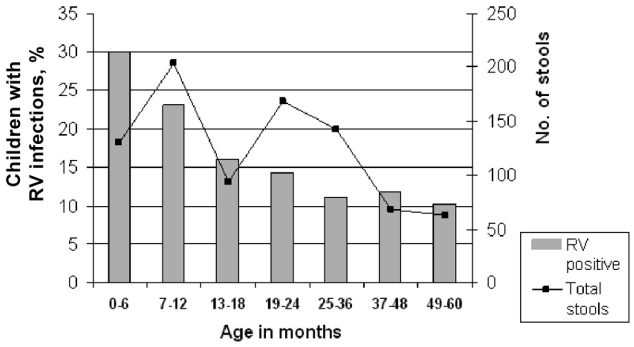

Figure 1. Age distribution for children with rotavirus (RV).

prevalence of rotavirus in infants aged 4–6 months (27.2%; 28 of 103 children) and the lowest in those aged <3 months (23.1%; 12 of 52 children). Furthermore, 78% of all children presenting with rotavirus diarrhea were <24 months of age. Rotavirus was detected all year round, with slightly increased seasonal peaks during the cooler, dryer months from October to March (Figure 2).

PAGE results. The RNA of rotavirus-positive specimens from diarrheal case and control subjects ($n = 170$) were all subjected to separation on polyacrylamide gels. Electropherotypes could be obtained from 55.8% of rotavirus-positive specimens (87 of 156) collected from diarrheal case subjects and from 35.7% (5 of 14) of those from age-matched control subjects. Long RNA migration patterns predominated in 80.4% of specimens (74 of 92), and 10 different long electropherotypes were noted. RNA profiles from the control group were all long electropherotypes. In addition, 6 distinct short electropherotypes ($n = 18$) and a small proportion of mixed patterns (2.2%) were detected.

Subgroup specificity. Subgroup specificity could be assigned to 146 of 170 specimens, with 22 specimens not reacting to any of the antibodies used and another 2 specimens having insufficient stool for testing. SGII specificity was found in 41 of 170 specimens, SGI in 37 of 170, and SGnon-I/non-II in 16 of 170. Surprisingly, SGI+II specificity was detected in 52 of 170 specimens, by far the predominant subgroup in specimens from Northwestern Nigeria.

VP7 and VP4 genotyping. The VP7 and VP4 genotyping results are summarized in Table 1. In total, 70% of specimens could be assigned a G genotype, and 67% could be assigned a P genotype. The number of specimens that could be genotyped included 51 and 48 strains from the rotavirus enzyme immunoassay–positive specimens with no visible first-round VP7 or VP4 products, respectively. In addition, 2 specimens with RT products could not be assigned a G type, and an additional specimen with a first-round VP4 product could not be P typed. Sequencing of these specimens revealed serotype G2s and P[6] genotypes.

Genotype G1 strains were predominant during the study period, accounting for 27% (46 of 170) of all strains circulating. Furthermore, mixed G genotype infections accounted for 26% (44 of 170) of circulating strains, and a total of 30% of strains could not be G genotyped using the primers described by Gouvea and colleagues [32]. Genotypes G8, G2, and G3 were detected at lower levels in 7.6%, 7.6%, and 1.7% of strains, respectively. During the study period, no single infections caused by genotype G4 or G9 strains were detected. Genotypes G4 and G9 occurred as mixed infections, with G9 occurring more commonly (7.0%: 12 of 170) than G4 (5.3%: 9 of 170). Similarly, P[6] was the predominant P genotype between 2002 and 2004, comprising 22% (38 of 170) of all circulating strains, and 21.8% (37 of 170) of strains bore mixed P genotypes. Genotypes P[8] and P[4] were detected in 17% and 5.8% of strains, respectively, and a total of 33% could not be P typed.

Genotyping results revealed that among the specimens collected from the nondiarrheic children, genotype G8 occurred more often (2 of 14 specimens) than G1 (1 of 14), G3 was not

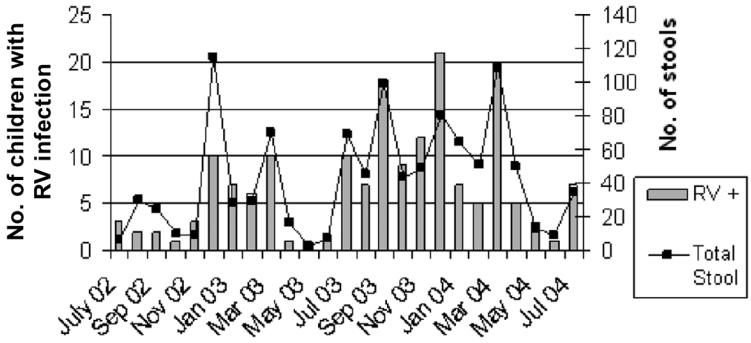

Figure 2. Monthly distribution of rotavirus (RV) infections.

Table 1. VP7 and VP4 Genotypes of Rotavirus-Positive Specimens Collected from Children with Diarrhea and from Age-Matched Control Subjects

	No. of specimens, by G genotype						
P genotype	G1	G2	G3	G8	Mixed	NT	Total
P[8]	26	0	0	0	2	1	29
P[6]	5	1	2	10	18	2	38
P[4]	1	9	0	0	0	0	10
Mixed	12	3	1	2	18	1	37
NT	2	0	0	1	6	47	56
Total	46	13	3	13	44	51	170

NOTE. Specimens were obtained from children <5 years old presenting to hospitals and clinics in 4 states in northwestern Nigeria between July 2002 and July 2004. NT, nontypeable.

detected, and 5 of 14 specimens from asymptomatic subjects were nontypeable. Genotypes G2, G4, and G9 occurred as mixed infections and accounted for 43% (6 of 14) of specimens from asymptomatic subjects. The VP4 genotype, P[6], was most frequently detected in specimens from asymptomatic subjects, whereas other P genotypes occurred as mixed infections.

Analysis of the mixed infections revealed multiple G and/or P types in 39% of specimens collected during the study period (Table 2). Two G and P types were identified in 20% and 17.6% of samples, respectively, and 3 G and P types were detected in 6.4% and 4.1% of specimens, respectively. Furthermore, in 1.7% of samples, 4 different G serotypes were observed. Rare P types, including P[10] and P[14], were also detected in mixed infections.

DISCUSSION

Nigeria is estimated to have one of the highest infant mortality rates in Africa, and the high incidence of childhood diarrhea is projected to account for >160,000 deaths annually in children <5 years of age. Approximately 20% of these deaths or 33,000 annually are estimated to be due to rotavirus-associated diarrhea [1]. The results generated in this study indicate that—unlike the situation in developed countries, where rotavirus infections are more common in children 9–15 months old [4]—the burden of rotavirus disease is highest in the youngest and most vulnerable (Figure 1). The detection of more rotavirus infections in children 4–6 months old than in those <3 months old may also reflect the protective effect from maternal rotavirus-specific antibodies during the first 3 months of life. Furthermore, rotaviruses were still detected in roughly 10% of diarrheal episodes in children >24 months old and probably represent secondary or tertiary infections in these settings rather than the first exposure event.

Analysis of the monthly distribution of rotavirus infection in northwestern Nigeria and determination of its seasonality was difficult. This may have been the result of poor diarrhea stool specimen collection during some months. However, rotavirus appears to infect children all year round, with slight increases during the cooler, drier winter months (Figure 2). These results were not unexpected and similar seasonality trends in Africa have been reported previously [5].

Almost no statistically significant differences were observed between variables from diarrheic and nondiarrheic children when analyzed (data not shown). Therefore, for the purpose of this study, no conclusions were based on the comparison of the 2 populations. However, there was a significant association between diarrhea and rotavirus infection ($P < .01$), and children with diarrhea were 2.5 times more likely to have been infected with rotavirus than those without diarrhea (odds ratio, 2.50; 95% confidence interval, 1.47–4.20).

Subgroup distribution among the Nigerian rotavirus positive strains was unusual. Genotype G1P[8] and G8P[6] strains were associated with all 4 subgroups, including SGI; G2P[6] and G3P[6] were detected among the SGI+II strains and most of the G nontypeable (G1, G1G9, and G8 nontypeable) strains; and the nontypeable strains displayed SGI specificity (data not shown). Iturriza-Gómara and colleagues [35] have demonstrated that strains serologically characterized as SGII, SGI+II, and SGnon-I/non-II genetically belong to the SGII lineage. This would partially explain the results observed and maintain partial genogroup correlation between SGII specificity and G1P[8] or G3P[6] genotypes. However, unusual combinations including SGI specificity and G1 and SGI+II specificity and G2 were detected, albeit at low levels. In addition, the nontypeable strains bearing SGI specificity may require additional investigation.

During the 2-year study period, globally common genotypes G1, G2, G3, G4, and G9 were detected in northwestern Nigeria. In addition, genotype G8 strains, frequently observed in Africa, were also reported. Genotype G8 has a wide distribution in animal species and has been reported in pigs, horses, and cattle [36]. In this study, it was the second most common genotype and the most prevalent in mixed infections. Although the G8 strains were not sequenced, animal-to-human transmission in the study area cannot be ruled out, because many of the inhabitants live in close proximity with their animals and often share a common source of drinking water. Adah and coworkers [37] have described elsewhere the close relationship between genotype G8 bovine and human rotavirus strains in Nigeria. In addition, analysis of study questionnaires revealed that ~24.7% of the children infected with rotavirus in the study had parents who owned animals and a further 18.2% of these infected children had contact with these animals (data not shown). The dusty and windy nature of northwestern Nigeria, especially in the dry season, could further increase the zoonotic transmission of animal rotaviruses and the transmission of human rotaviruses from environmental sources via aerosols to

Table 2. Mixed G and P Genotypes of Rotavirus-Positive Specimens Collected from Children with Diarrhea and from Age-Matched Control Subjects

	No. of specimens, by P genotype											
G genotype	P[6]	P[8]	P[4]P[6]	P[4]P[8]	P[6]P[8]	P[6]P[10]	P[6]P[14]	P[8]P[14]	P[4]P[6]P[14]	P[6]P[8]P[10]	NT	Total
G1	0	0	0	5	5	5	0	0	0	1	0	16
G2	0	0	3	0	0	0	0	0	0	0	0	3
G3	0	0	0	0	1	0	0	0	0	0	0	1
G8	0	0	0	0	0	1	1	0	0	0	0	2
G1G2	1	1	0	0	0	0	0	0	0	0	0	2
G1G3	0	0	0	0	0	0	0	0	0	0	2	2
G1G8	10	0	0	0	0	0	0	0	0	0	2	12
G1G9	0	1	0	0	0	0	0	0	0	0	1	2
G2G3	0	0	0	0	0	0	0	0	1	0	0	1
G2G4	1	0	0	0	0	2	0	0	0	0	0	3
G2G8	0	0	2	0	0	0	0	0	0	1	0	3
G2G9	1	0	0	0	0	0	0	0	0	0	0	1
G3G9	0	0	0	0	1	0	0	0	0	0	0	1
G4G8	0	0	0	0	0	1	0	0	0	0	0	1
G4G9	1	0	0	0	0	1	0	0	0	0	0	2
G1G2G4	0	0	0	0	0	0	0	0	0	1	0	1
G1G2G8	1	0	4	0	0	0	0	0	1	0	0	6
G1G2G9	1	0	0	0	0	0	0	0	0	0	0	1
G1G3G8	0	0	0	0	0	0	0	0	0	0	1	1
G1G3G9	1	0	0	0	0	0	0	0	0	0	0	1
G2G4G9	0	0	0	0	0	0	0	0	0	1	0	1
G1G2G8G9	1	0	1	0	0	0	0	0	0	0	0	2
G2G4G8G9	0	0	0	0	0	0	0	0	0	1	0	1
NT	0	0	0	0	0	0	0	1	0	0	0	1
Total	18	2	10	5	7	10	1	1	2	5	6	67

NOTE. Specimens were obtained from children <5 years old presenting to hospitals and clinics in 4 states in northwestern Nigeria between July 2002 and July 2004. NT, nontypeable.

children living within the area. Therefore, high detection levels of both the bovine common serotype G8 strains and mixed infections should not be unforeseen.

An unusual observation noted in this study was the number of mixed infections in which 3 and 4 P or G genotypes were detected (Table 2). The proportions of mixed infections detected by PAGE and RT-PCR were also discrepant. This difference may be due in part to the different detection limits for the 2 analysis techniques. Early studies determined that the PAGE technique gave similar sensitivities for both electron microscopy and ELISA, roughly 10^5 to 10^6 particles per milliliter [28], whereas the RT-PCR detection limit has been shown to range from 10^1 to 10^3 particles per milliliter [38]. It is also reasonable to expect, based on variations in infective dose, intrinsic strain characteristics, and gut physiology, that the respective concentrations of different rotavirus strains in a mixed infection may differ. Although some of the mixed infections detected may be the result of incorrect primer binding due to the genetic drift in rotavirus genomes, these results should not be entirely unanticipated considering the limited access to clean water, the absence of sanitation, and poor socioeconomic conditions. These results may also highlight the multitude of enteric pathogens to which children in these African settings must be exposed; given this exposure, coupled with the added burden of malnutrition, the high rate of diarrhea-associated mortality estimated for Nigerian children should not be surprising.

The high rate of mixed rotavirus infections is a very common finding in Northern Nigeria. Studies by Adah et al [39], Pennap et al [25], Audu et al [19], and Steele et al [40] have all reported "mosaic" rotavirus strains with G1G3 specificity. In a later study in Nigeria, Adah et al [41] also reported the predominance of the mosaic rotaviruses with G1G2. In this study, mixed infections with the predominant strains G1, G2, and G8 and additional strains G4 and G9, not identified in single infections, were observed. It is important to note that the other studies have used different techniques, including antigenic analyses with monoclonal antibodies [25] and RT-PCR typing with various primers [39]. Therefore, though these mixed infections should be further studied to elucidate this phenomenon in Nigerian children, it seems likely based on evidence reported

elsewhere that these mixed infections are a real characteristic of rotavirus infections in Nigeria, rather than an artifact of typing.

Genotype G9 strains were detected only in conjunction with genotypes G1–G4, further extending the global distribution of this genotype and supporting the promotion of G9 as the fifth most important genotype of strains worldwide. During this study, G9 strains were not detected in children >24 months old (data not shown). This is contrary to a previous study in Nigeria that found G9 infections in slightly older children [40] and supports the premise that genotype G9 strains were only recently introduced into African populations [42] (unpublished observations). G9 strains have subsequently become established within Africa and may prove to circulate cyclically, similarly to G2 or G4 strains.

The predominance of genotype P[6] in Africa is not novel, having been detected in Ghana [43], in Malawi [44], and in several studies in Nigeria [19, 25, 39, 41]. Likewise, P[6] was detected in 22% of specimens and in a further 20.5% of mixed infections with additional P genotypes during the study period (Table 2). Reports of new or unusual G genotypes associated with P[6], including G8P[6] in Malawi [44], G9P[6] in South Africa (unpublished observations) and Ghana [45], G10P[6] in Ghana (unpublished observations), and G12P[6] in South Africa [46], suggest that reassortment of these unusual G types with the P[6] genotype may be one of the mechanisms by which these viruses enter naive populations, especially in Africa. After the introduction of the unusual P[6]GX genotype, the viruses undergo reassortment with commonly circulating strains, probably bearing the P[8] genotype and more ecologically suited for infection of human hosts.

In this study, P[10] strains were detected in mixed infections (Table 2) and were found in a high proportion of asymptomatic infections (data not shown). Previously, P[10] strains have been identified sporadically in Ghana, Kenya, Cameroon, and South Africa [47]. Similarly to the P[10] first detected in Indonesia associated with G8 and "supershort" RNA electropherotype [48], the P[10] in this study was found in association with P[6], G1, G8, and mixed G and P genotypes. Another rare P type, P[14], was also detected in 4 children with mixed infections and associated with asymptomatic infection (data not shown). These results are also comparable to reports of asymptomatic P[14] detection in Italy by Arista et al [49]. Further investigation of these rare P genotypes will be required to confirm their presence in mixed infections and to determine their genetic character.

The high proportion of nontypeable strains is a common feature of rotavirus characterization in most developing countries, where up to 30% of strains may remain untyped [50, 51]. These strains may represent common strains with accumulated point mutations in the currently used primer binding sites; animal rotaviruses including G5, G6, G10, and G12; or new unidentified rotavirus strains. Sequence analysis and the constant monitoring of genetic drift and shift would aid researchers in the developing world in the typing of circulating rotavirus strains. Collaborations between laboratories in First and Third World settings may also help promote these aims.

Although the introduction of either oral, live attenuated rotavirus vaccine into the Expanded Program on Immunization schedules of African countries may take more than a few years to implement, data on circulating strains may serve 2 purposes. The first will be to enlighten and educate African government and health officials that rotavirus is a serious health issue in their respective countries and the second will be to document strains diversity before vaccine introduction so that questions surrounding escape mutants and strain replacement can be more readily tackled. Additional studies investigating the burden and cost of rotavirus disease, however, will be required to expedite the introduction of rotavirus vaccines to children of the developing world, who would clearly benefit from these interventions.

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